Algal Research 17 (2016) 132–141

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Molecular exploration of algal interaction between the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Alexandrium tamarense*

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ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form 22 March 2016
Accepted 20 April 2016
Available online 12 May 2016

Keywords:
Diatom
Dinoflagellate
Algal interaction
Allelopathy
Transcriptome pervasive

ABSTRACT

The succession of dominant phytoplankton species plays an important role in harmful algal blooms. However, the molecular mechanism of algal succession remains largely unclear, including the most commonly occurring diatom/dinoflagellate succession. Here we investigated the responses of the diatom *Phaeodactylum tricornutum* during mixed culture with the potentially toxic dinoflagellate *Alexandrium tamarense*. The growth of *P. tricornutum* was significantly inhibited within 24 h in mixed culture. Organelles such as the chloroplasts and mitochondria of *P. tricornutum* were severely damaged. Transcriptional responses in *P. tricornutum* were revealed by RNA-seq. Genes involved in glycolysis, TCA cycle, β-oxidation, carbon fixation and oxidation phosphorylation were downregulated, indicating the inhibition of energy metabolism. Several genes associated with check points and cell cycle were also downregulated, suggesting the suppression of DNA replication and cell division. Taking into account the upregulation of genes involved in endocytosis and transporter ABCB1, *P. tricornutum* could perceive certain allelochemicals released from *A. tamarense*, which played a role in their interaction. Additionally, a family of leucine-rich repeat receptor-like kinases was upregulated, suggesting that flagellin-sensitive mediated cell-cell interactions are responsible for toxic effect other than allelopathy. Here we showed a molecular overview of interactions between diatom and dinoflagellate, thereby provide molecular insight into the unraveled mechanisms on the effects of toxic species on non-toxic species.

1. Introduction

Among primary producers, pervasive phytoplankton plays a pivotal role in photoautotrophic production for oceanic carbon cycle and facilitates the cycling of biomass and energy fluxes in marine ecosystems [1]. Uncontrolled proliferation of algal blooms depletes the dissolved oxygen and some blooms threaten zooplankton as they secret noxious toxins. Several factors have been proposed to influence the formation of harmful algal blooms (HABs), among them climatic conditions such as water stratification has been considered as the significant factor in HABs formation [2–4]. The alteration of nutrients ratio, such as nitrate and phosphate in coastal waters caused by nutrient discharges in aquatic environments may be another important cause of HABs [5]. Nutrient abundance in seawater have been linked to the formation of HABs in the Tyrrhenian Sea [2] and the Gulf of California [6]. Moreover, previous studies showed that allelochemicals could also play a role in the formation of HABs [7].

The dinoflagellate genus *Alexandrium* accounts for a large proportion of HAB species, and its allelopathic activity have been reported to play an important role in dominance of *Alexandrium* spp. (*Alexandrium tamarense* and *Alexandrium minutum*) [8]. Previous studies have shown that *Alexandrium* spp. can secret lytic compounds that lyse other algae and heterotrophic protists within a short period of time [9]. Mixed growth experiments of *A. tamarense* with other planktonic algae under nutrient-rich conditions revealed that have shown that all the target algae were negatively affected by *A. tamarense* strain Alex2 [10]. These findings indicated that the succession of dominant phytoplankton species was not only restricted by competing nutrient availability, but also by allelopathic deterrence With the advent of resting cyst stage formation under suboptimal growth conditions, life cycle of *Alexandrium* spp. potentially enhance the bloom formation. Thus, this

Abbreviations: HABs, harmful algal blooms; KEGG, kyoto encyclopedia of genes and genomes; KO, KEGG orthology; TEM, transverse electro-magnetic; UPLC-MALDI-MS, ultra performance liquid matrix-assisted laser desorption/ionization mass spectrometric; ABC, ATP-binding cassette; FLS2, flagellin-sensitive 2.

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http://dx.doi.org/10.1016/j.algal.2016.04.019

resting stage can provide *Alexandrium* with a competitive advantage over other populations that cannot persist under optimal growth conditions. The subsequent germination and migration of cells into surface waters can also ensure their transport throughout a region [11].

However, the precise molecular mechanisms underlying the interactions between diatoms and dinoflagellates are poorly understood. Here, we attempt to investigate the molecular responses of the marine diatom *Phaeodactylum tricornutum* [12], during mixed culture with a lytic strain of *A. tamarense*. By analyzing the effects of *A. tamarense* during mixed culture, we were able to reveal key factors that may be involved in the interaction between diatoms and toxic dinoflagellates.

2. Materials and methods

2.1. Algal growth conditions

Strains CCMP2561 of *P. tricornutum* (obtained from the National Center for Marine Algae and Microbiota) and ATDH23 of *A. tamarense* (isolated from the East China Sea) were used in these experiments. Both *P. tricornutum* and *A. tamarense* were cultured in f/2-Si medium [13] which were filtered through 0.22 μm filters. The cultures were cultivated under controlled conditions at 20 °C under white light (200 μmol m^{-2} s^{-1}) with a 12:12 h light/dark regime.

The interaction analyses between *P. tricornutum* and *A. tamarense* were performed in triplicates, with each in 2.0 L flasks with 1.6 L cultures. Mixed cultures of *P. tricornutum* and *A. tamarense* cells during the exponential phase were used and mono culture was used as control. In the mixed cultures, *P. tricornutum* and *A. tamarense* were mixed together and supplemented with fresh f/2-Si medium to 1.6 L. In mixed culture, initial cell density of *P. tricornutum* and *A. tamarense* was 1.0×10^6 cells mL^{-1} and 3.0×10^3 cells mL^{-1} [14,15], respectively.

In mono culture, *P. tricornutum* cells were immediately supplemented with fresh f/2-Si medium to 1.6 L. Additionally, an extra 8.16 mL of f/2-Si stock solution was added to both the cultures to ensure nutrient-rich conditions.

The effect of cell-free *A. tamarense* supernatant on *P. tricornutum* was evaluated in 250 mL flasks with 200 mL of culture. Cell-free supernatant from exponential-phase *A. tamarense* cells were harvested (4400 rpm, 15 min, 4 °C). Exponential-phase *P. tricornutum* cells were cultured with cell-free supernatant of *A. tamarense* with the initial cell density of 1.0×10^6 cells mL^{-1}. *A*1.02 mL of f/2-Si stock solution was added to the treatment and control cultures.

Cells grown in mixed culture and cell-free *A. tamarense* supernatant were collected at approximately same time each day in triplicates. Cell numbers of *P. tricornutum* and *A. tamarense* were counted with Brightline hemocytometer under light microscope (Olympus) every day. Algal cell concentration (cells mL^{-1}) was calculated using the method previously described [16].

2.2. Separating diatom cells from mixed cultures

P. tricornutum was separated from mixed culture grown for 24 h using PET filters (8.0 μm) to intercept *A. tamarense*. Mixed cultures (1.6 L) were filtered for 5–6 times and purity of diatom fraction were confirmed by microscopy. *P. tricornutum* cells were harvested (4400 rpm, 10 min, 4 °C), resuspended in fresh medium (1 mL) and 20 μL of resuspended solutions were examined under a light microscope to ensure cell concentration of *A. tamarense* was <500 cells mL^{-1}. Diatom cells from both mixed and mono cultures were collected and immediately frozen with liquid nitrogen, and stored at −80 °C until RNA extraction.

2.3. RNA-seq analysis of P. tricornutum

P. tricornutum samples from mixed and mono culture in triplicate were used for total RNA extraction. RNAiso Plus (TaKaRa) was used in accordance with the manufacturer's instructions, with a slight modification in which volume of lysis buffer was increased to improve RNA yield and integrity. RNA integrity was assessed on the Bioanalyzer 2100 system using RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). Total RNA from diatom isolated grown in mixed cultures (in triplicate) was pooled and used as the treated sample. Similarly, total RNA from three biological replicates of mono cultures were also pooled and used as the control sample. The library preparations of treated and control sample were sequenced on a HiSeq2000 (Illumina).

The sequenced reads extracted from the HiSeq2000 were quality checked using RSeQC [17]. Clean reads for each sample were aligned against the *P. tricornutum* genome and annotated according to (http://genome.jgi-psf.org/Phatr2/Phatr2.download.ftp.html) the TopHat2 algorithm [18]. The abundance of each transcript in each sample was represented by RPKM and estimated using HTseq (http://www-huber.embl.de/users/anders/HTSeq/). The differential diatom gene expression between mixed and mono cultures were analyzed by DESeq [19]. Corrected *P*-value of 0.05 was set as the threshold for statistical significance. To assign genes into pathways, KOBAS [20] was used to annotate genes into the *P. tricornutum* KEGG maps and KO maps, and gene lists were manually compiled for the major pathways. The organelle localization for the gene products was confirmed with annotations from JGI, NCBI and ENSEMBL. If no targeting was found, the localizations were predicted with a TargetP 1.1 server [21] with 'specificity >0.95' cutoffs and both plant and non-plant modes, with Cell-PLoc [22] in both Euk-mPLoc and Plant-PLoc.

2.4. Ultrastructural analysis of algal cells

Cells of both mixed and mono cultures were collected by centrifugation at 4400 rpm for 10 min at 4 °C and the supernatant discarded. Samples were then rinsed three times by using 1 × PBS and collected by centrifugation at 5000 rpm for 3 min. Ultrathin sections were prepared according to the method of Yang [16]. The stained sections were examined with a TECNAI-10 transmission electron microscope (PHILIPS, Netherlands).

2.5. Extraction and analysis of extracellular toxins from A. tamarense

The extracellular toxins of *A. tamarense* were extracted as described elsewhere [15], with slight modifications. Briefly, exponential-phase mono cultures of *A. tamarense* were centrifuged at 4400 rpm for 10 min at 4 °C, and the supernatant was collected and filtered through a 0.22 μm filters (Millipore). After pH was adjusted with 1 M NaOH to 10–11, the preparation was left to stand for 1 h for precipitation. Thereafter, precipitates were collected by centrifugation (4400 rpm, 10 min, 4 °C). The precipitates were dissolved in 1 M HCl and dialyzed (membrane cut-off 1 kDa) for 3 days at 4 °C. The dialysates were lyophilized and dissolved in 1 mL of methanol to obtain crude extracellular toxins.

The extracellular toxins were analyzed by UPLC-MALDI-MS according to previous investigations [23]. The resulting compounds were searched in ChemSpider database (http://www.chemspider.com/).

3. Results and discussion

3.1. Growth of P. tricornutum and A. tamarense affected by mixed culture

The growth of *P. tricornutum* was inhibited in the mixed cultures by the presence of *A. tamarense* compared with control *P. tricornutum* culture (Fig. 1A). The growth of *P. tricornutum* was significantly suppressed from the first day of inoculation ($P < 0.05$), and the cell concentration was dropped by approximately 50% of the control culture. The growth trend of *P. tricornutum* in the mixed cultures was stagnated, their growth ceased thereafter. The previous studies showed the negative

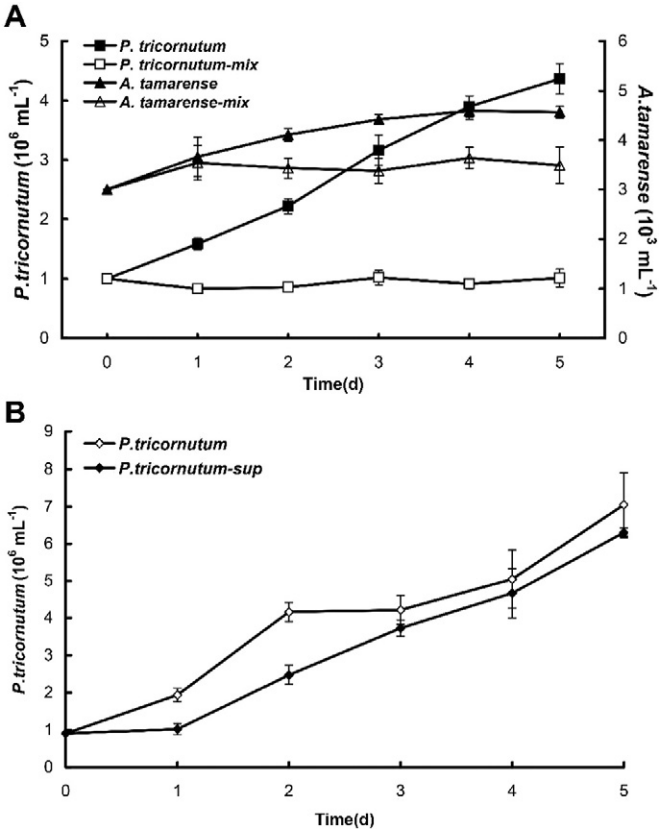

Fig. 1. Growth characterization. (A) *P. tricornutum* and *A. tamarense* in mixed cultures; (B) the effect of *A. tamarense* cell-free supernatant. *P. tricornutum* and *A. tamarense* were grown as a mixed culture, with their individual cultures as controls.

impact of *Alexandrium* on diatom growth in mixed culture [10,24]. The results also showed that under nutrient-rich conditions, the diatom species were dramatically affected when co-cultured with *A. tamarense* strain Alex2, which could produce lytic extracellular compounds. The interaction between some *Alexandrium* spp. and algae were also found to be caused by the allelochemicals in the cell-free cultures and the toxic affects were not due to the cellular paralytic shellfish poisoning toxins [8,15]. Our results suggest that allelochemicals of *A. tamarense* could be one of the factors that caused *P. tricornutum* suppression in mixed cultures, consistent with the previous studies. The growth of *P. tricornutum* was delayed by the cell-free supernatant of *A. tamarense* in the early phase (Fig. 1B). A previous study showed that growth of *P. tricornutum* was inhibited by ethyl 2-methyl acetoacetate (EMA), a novel allelochemical [25]. *P. tricornutum* suffered oxidative stress upon exposure to EMA, with characteristic decreased cellular content and overall physiological characteristics. Furthermore, we characterized the potential compounds in cell-free supernatant of *A. tamarense* by UPLC-MALDI-MS, including polypeptides, esters and organic acids (Table 1). The precise functions of these compounds, however, remain unclear. Previous studies have shown that allelochemicals from *A. tamarense* were complicated and could consist of polysaccharides, proteins or esters [26,27]. However, our findings revealed that there was no significant reduction in *P. tricornutum* growth, when cultured with cell free extract. We hypothesize that reduced toxicity of cell-free supernatant of *A. tamarense* night be due to the oxidative decomposition of active substances in the culture medium, since the main components of the cell-free supernatant were determined to be unstable and degradable lipid or protein polypeptides, etc.

Similarly, the cell concentration of *A. tamarense* in mixed cultures was also slightly affected on third day, whereas the control cells did not show such growth aberrations as they lacked the competitor in the culture. In the co-culture of *Prorocentrum donghaiense* and *A. minutum*, both algal species experienced a drop in cell concentration compared to their corresponding control cultures [8,15]. The decreased growth of *A. tamarense* observed in this study might be caused by nutrient competition between the organism or other factors such as lysis of *P. tricornutum* cells.

Table 1
Compounds detected in the cell-free supernatant of *A. tamarense*.

ChemSpider ID	Molecular formula	Average mass (Da)
Organic acid		
10144400	$C_{28}H_{25}N_5Na_4O_{20}S_6$	1035.869019
8074180	$C_{61}H_{114}N_6O_{11}$	1107.592041
9041498	$C_{54}H_{84}O_{36}S_6$	1501.613037
8008855	$C_{28}H_{21}K_5N_6O_{16}S_4$	1021.248413
Ester		
10155355	$C_{54}H_{32}F_{18}N_{12}O_{12}$	1382.876343
Polypeptide		
8255503	$C_{57}H_{110}N_{14}O_{11}$	1167.571045
23337116	$C_{60}H_{105}N_{23}O_9$	1292.62439
9084654	$C_{65}H_{115}N_{11}O_{13}S$	1290.739502
Others		
13176992	$C_{20}H_{30}N_{10}O_{20}P_6S_6$	1108.739746
21191792	$C_{49}H_{43}Cl_6N_3O_4P_3Ru$	1144.590942
24735577	$C_{36}H_{50}Mo_4N_4O_{11}$	1098.562378

Results from UPLC-MALDI-MS were searched in ChemSpider and presented with their ChemSpider IDs.

3.2. Subcellular ultrastructure of *P. tricornutum* and *A. tamarense* affected by mixed culture

As shown in Fig. 2, there were no unusual ultrastructural differences observed between *A. tamarense* grown in mixed culture and control medium (Fig. 2A and B). The cellular structures of *A. tamarense*, including the nucleus, chloroplast, mitochondrion, were clearly identifiable from both the control and mixed cultures.

However, the ultrastructure of *P. tricornutum* grown in mixed culture with *A. tamarense* was severely affected within 24 h of (Fig. 2D and F). The chloroplasts and mitochondria were clearly identifiable and organized in control *P. tricornutum* cells (Fig. 2C and E). On the other hand, the chloroplast membranes were blurred or partially ruptured and the thylakoids had disintegrated; and the borders among them were indistinguishable in *P. tricornutum* from mixed cultures. The mitochondria were deformed and the mitochondrial cristae were degraded. Similarly, Wang and Zheng [28] observed the negative impact of copper treatment on sub-cellular organelles in *P. tricornutum*. These findings indicated that co-incubating with *A. tamarense* induced an environmental stress that affected the normal cellular function of *P. tricornutum*.

3.3. Analysis of transcriptional changes in *P. tricornutum* by RNA-seq

Each sample was sequenced by Illumina (Fig. 3A). The complete dataset covered 94% of the predicted genes. In both control (PtCtr) and mixed cultures (PtAt), 9502 genes were expressed (Fig. 3B). Additionally, 2735 genes were differentially expressed at statistically significant levels (Fig. 3C), which included 1545 upregulated genes and 1190 downregulated genes. KEGG annotated results showed that these differentially expressed genes were categorized into the cell cycle, signal transduction, nucleic acid metabolism and carbon metabolic pathways including photosynthesis, oxidative phosphorylation, glycolysis, and the TCA cycle (Figs. 4, 5, 6; Table 2).

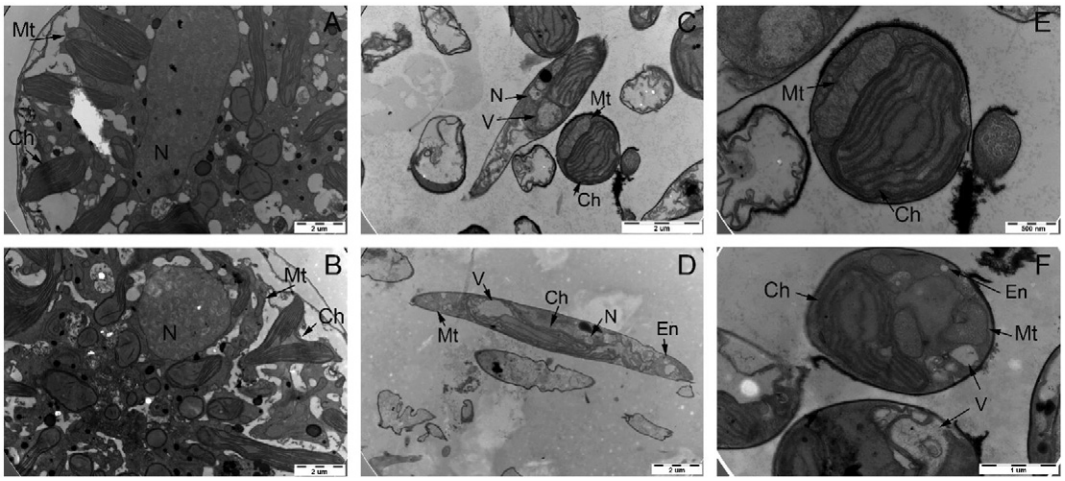

Fig. 2. Subcellular structure of *P. tricornutum* and *A. tamarense* after mixed culture for 24 h. (A) and (B): the control and mixed cultures of *A. tamarense*, respectively; (C) and (E): control *P. tricornutum*; (D) and (F): *P. tricornutum* in mixed cultures. N: nucleus; Mt: mitochondrion; Ch: chloroplast; V: vacuole; En: endosome.

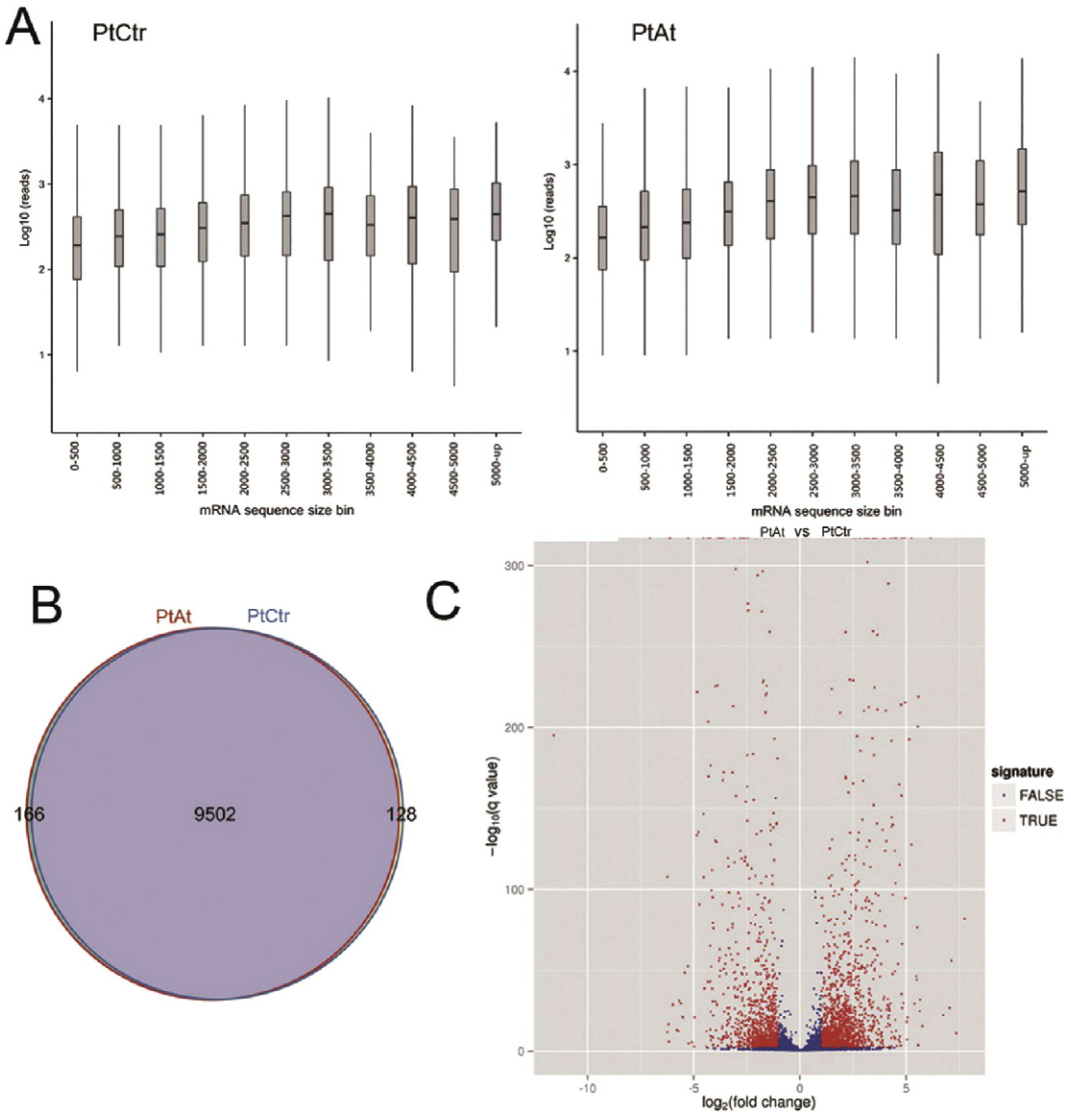

Fig. 3. Diagram of mRNA sequence analysis in *P. tricornutum* from control (PtCtr) and mixed cultures (PtAt). (A) Distribution of different mRNA sequence size. (B) The amount of genes. (C) The identification of differentially expressed genes.

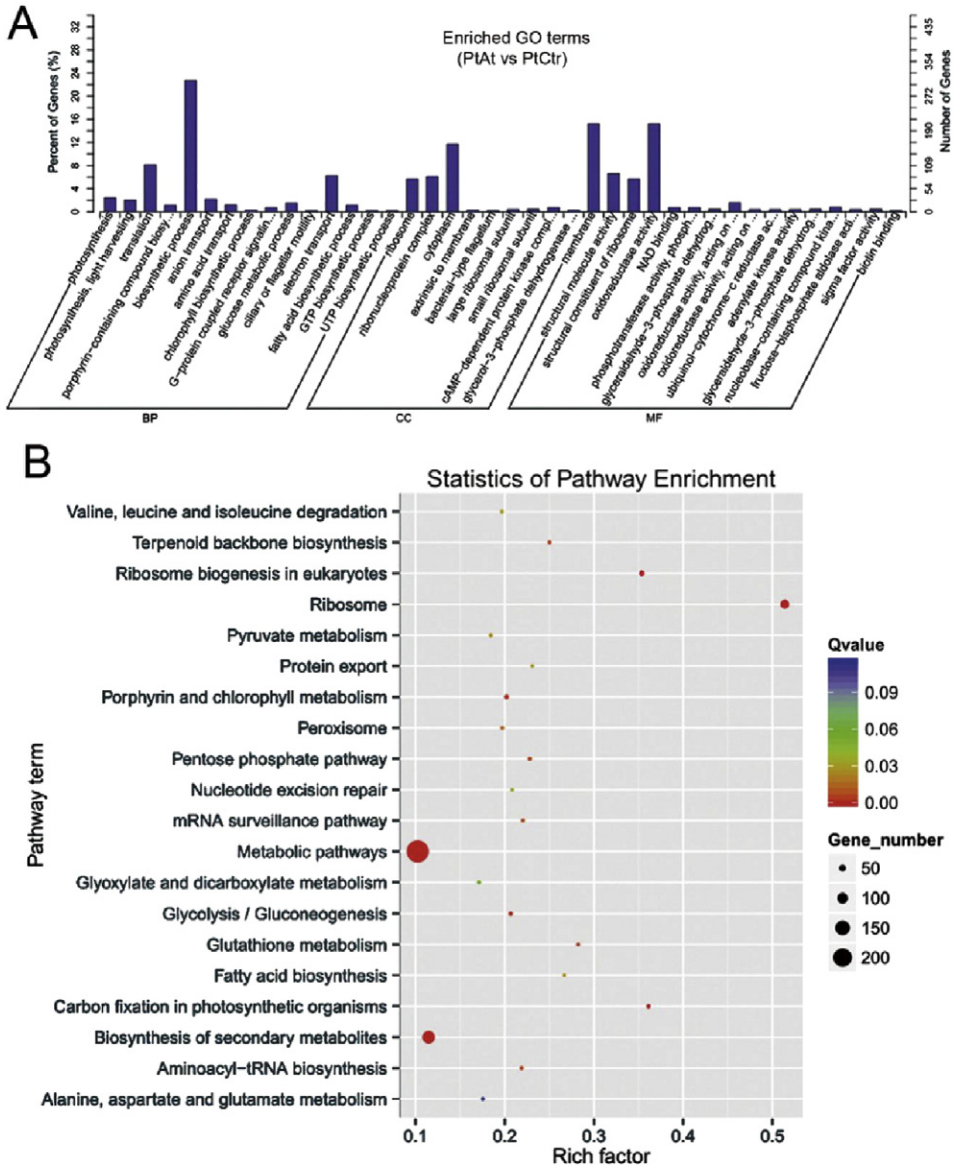

Fig. 4. Enrichment of GO terms and the KEGG pathway in *P. tricornutum*. (A) GO terms; (B) KEGG pathway.

3.4. The processes associated with the interaction between P. tricornutum and A. tamarense

3.4.1. Sensing

The ABC (ATP-binding cassette) transporter superfamily proteins are vital for the import of nutrients or the export of drugs, toxins and other xenobiotics [29]. It has been shown that inhibition of ABC transporter affects the export of organic anions in diatoms [30]. This finding suggests that the ABC transporter could play a critical role in the extrusion of xenobiotics from diatoms. Our results showed that mixed culture with *A. tamarense* induced the upregulation of several ABC transporters in *P. tricornutum*. Among the four identified ABC transporters, the annotation of Phatr2_1613 indicated a characteristic of ABCB1, also known as multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp).

Increased activity and expression level of P-glycoprotein when exposed to xenobiotics or other natural substrates revealed that P-glycoprotein could prevent uptake and accumulation of hazardous environmental xenobiotics or toxic chemicals [31,32,33]. The upregulation of P-glycoprotein in *P. tricornutum* co-cultivated with *A. tamarense* revealed that *P. tricornutum* cells were responding to allelopathy of *A. tamarense*, thus enhanced the efflux of toxic allelochemicals and other compounds out of the cell that reduced the cell damage.

3.4.2. Interesting possibility of flagellin-sensitive 2 (FLS2) proteins mediated algal interactions

FLS2 is a leucine-rich repeat (LRR) receptor-like kinase involved in flagellin perception response of pathogen-plant interactions [34]. A conserved 22-amino acid peptide called flg22 that represents the flagellin was previously shown to have a specific interaction with FLS2. This peptide stimulated the expression of associated resistance genes [35] that showed the key role of FLS2 in pathogen recognition and defense response. A total of 6 LRR receptor-like serine/threonine-protein kinases were annotated in *P. tricornutum*, and all of these kinases were upregulated by 3- to 5-fold in the mixed culture with *A. tamarense*, with an exception of Phatr2_1196 that dramatically upregulated by 22.69-fold (Dataset 1 in the online version at http://dx.doi.org/10.1016/j.algal.2016.04.019.). Intriguingly, BLASTP homology search showed that these kinases were similar to some FLS2 proteins from higher plants; for instance, Phatr2_1196 showed similarity to *Brassica carinata* flagellin-sensing 2-like protein (37% identity), *Arabidopsis thaliana* and

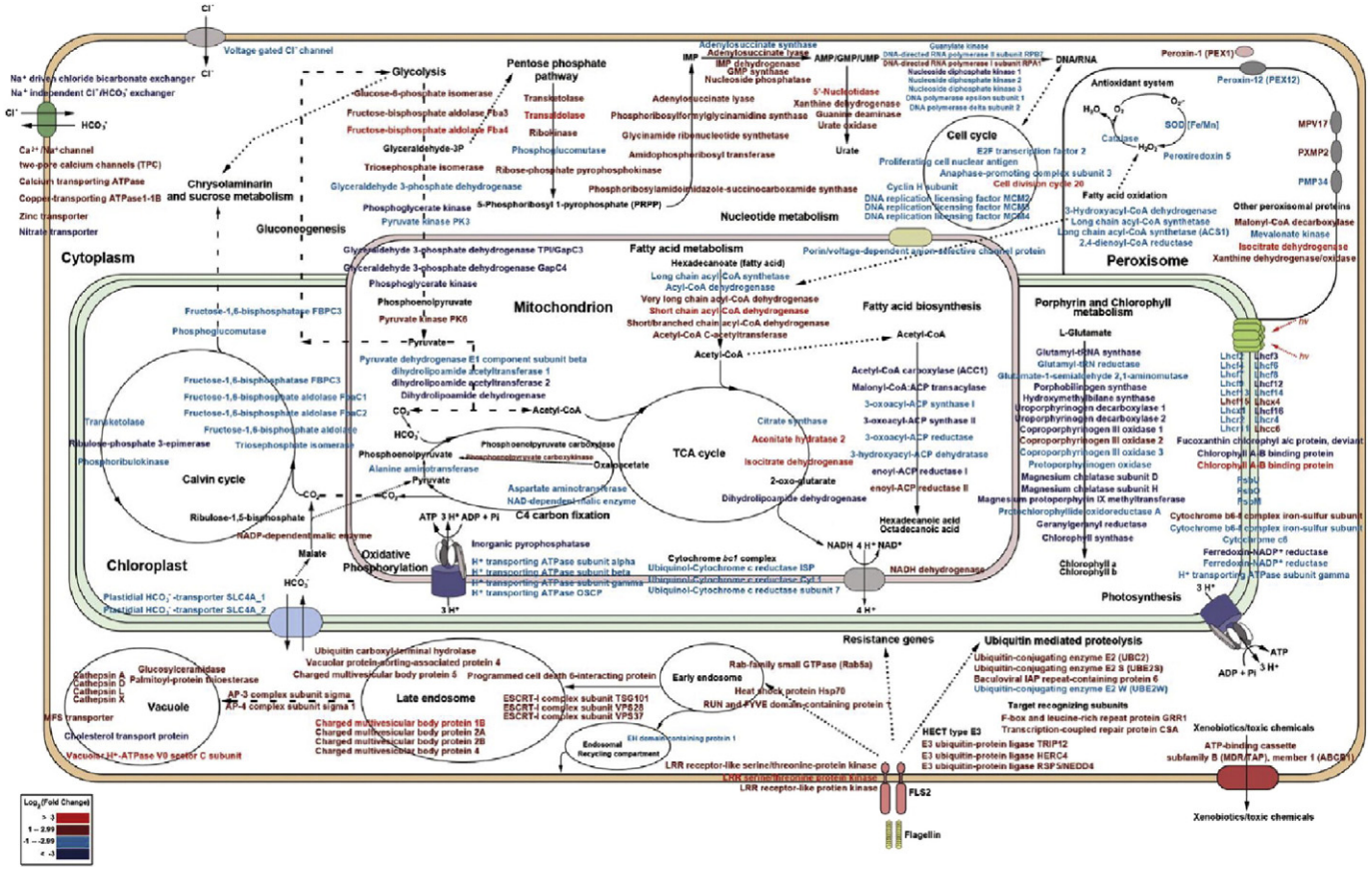

Fig. 5. Cellular metabolism map of *P. tricornutum* during mixed culture with *A. tamarense*. The color scale represents the downregulation (blue) and upregulation (red) of genes.

Aegilops tauschii LRR receptor-like serine/threonine-protein kinase FLS2 (35 and 37% identity, respectively), and also similarity to LRR-GTPase of ROCO family from the brown alga *Ectocarpus siliculosus* (38% identity), implied that they might be involved in FLS2-mediated cell-cell interactions. Therefore, we speculate that cell-cell interaction pathway has been existed between *A. tamarense* and *P. tricornutum* via flagellar interaction, which is distinct from the well-known allelopathy effect. We further hypothesize that dinoflagellate flagellin is recognized by diatom FLS2, and it stimulates the transduction of signal pathways, thereby inducing the expression of resistance genes and/or the response of the immune repertoire. The upregulation of LRR receptor-like kinases suggested an FLS2-mediated defense mechanism could play a role in response to *A. tamarense* and reduced the susceptibility to flagella invasion.

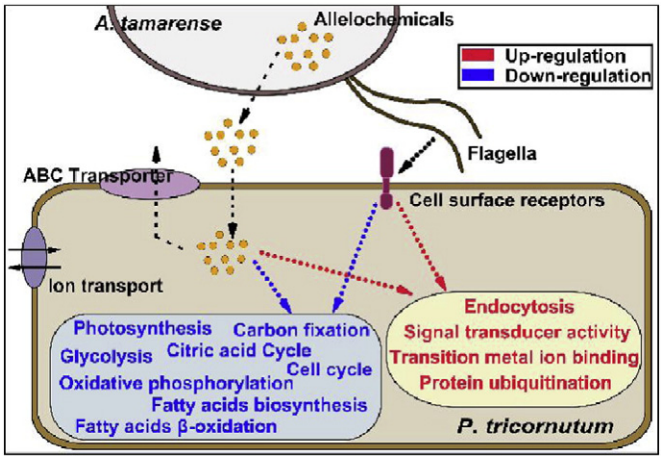

Fig. 6. Simplified overview of the molecular mechanisms of the *P. tricornutum*-*A. tamarense* interaction. The blue and red arrows represent the inhibition and stimulation of processes in *P. tricornutum*, respectively.

Flg22 and FLS2 binding resulted in ligand-induced endocytosis with internalization and degradation of FLS2 in *Arabidopsis* [36]. Our results also revealed that genes associated with endocytosis were upregulated under mixed culture. Ten genes involved in the formation of endosomes, especially the late endosomes, were dramatically upregulated. Among them, Rab5 (Phatr2_51511), the critical regulator during endosome biogenesis was found to be upregulated. Rab5 has recently been shown to involve in endocytic trafficking of activated FLS2 receptors and sorting the receptors into late endosome and multivesicular bodies [37]. Two E3 ligases (Phatr2_14177; Phatr2_14345) were upregulated in our results, and they were annotated as RSP5/NEED4. Two U-box E3 ligases were recruited to FLS2 receptor complex to direct the polyubiquitination of FLS2 and also to promote the ligand-induced degradation in *Arabidopsis* [38]. Moreover, many other components involved in the ubiquitin-mediated proteolysis pathway, such as the subunits of the E2 ligase, HECT-type and multi-subunit RING-finger type E3 ligase, were largely upregulated under mixed culture with *A. tamarense*. Congruently, several genes encoding the vacuole located proteins were also upregulated, such as CLN7, vacuolar membrane protein, and vacuolar protease cathepsins. The upregulation of vacuole-associated proteins might correlate with ligand-induced endocytosis and/or response to allelochemicals.

Taken together, the proposed FLS2-mediated cell-cell interaction between *P. tricornutum* and *A. tamarense* unraveled a different mechanism distinct from the well-known allelopathy effects, and the function of

Table 2
Fold changes in the expression of genes encoding enzymes involved in primary metabolism.

Transcript ID	Annotation	RPKM (PtAt)	RPKM (PtCtr)	Log2(PtAt/PtCtr)	*P*-value
26970	NADH dehydrogenase	408.78	131.67	1.64	3.71E−80
42018	Ferredoxin-NADP+ reductase	62.74	278.89	−2.14	1.09E−32
21183	Inorganic pyrophosphatase	47.70	386.88	−3.0113	5.45E−160
20143	Long-chain acyl-CoA synthetase	6.19	16.93	−1.4432	3.66E−05
54926	Acetyl-CoA carboxylase	4.13	35.41	−3.09	1.11E−40
37652	Malonyl-CoA:ACP transacylase	5.71	435.07	−6.24	8.73E−110
23059	Aspartate aminotransferase	25.88	66.28	−1.35	5.18E−07
54082	NAD-dependent malic enzyme	16.63	78.82	−2.24	8.75E−18
34010	Alanine aminotransferase	136.50	442.29	−1.69	1.09E−54
55018	Phosphoenolpyruvate carboxykinase	103.79	46.57	1.16	1.82E−11
53935	Ribulose-phosphate 3-epimerase	6.55	82.86	−3.65	9.11E−17
10208	Phosphoribulokinase	282.36	834.30	−1.55	6.58E−69
22993	Fructose-1.6-bisphosphate aldolase	45.61	190.23	−2.05	7.94E−33
9359	Fructose-1.6-bisphosphatase	4.85	22.56	−2.21	3.14E−04
32708	Phosphoglucomutase	2.82	16.92	−2.58	7.84E−06
23598	Glyceraldehyde-3-phosphate dehydrogenase	18.90	88.70	−2.22	1.13E−15
29157	Phosphoglycerate kinase	14.58	246.04	−4.07	5.71E−78
54998	Pyruvate kinase	5.18	16.70	−1.68	9.27E−04
23850	Dihydrolipoamide acetyl transferase	0.39	13.31	−5.08	1.58E−06
54477	ATP-citrate synthase	43.33	103.41	−1.25	5.31E−20
26290	Aconitate hydratase 2	605.73	31.51	4.27	0.00E+00
23913	Acetyl-CoA C-acetyltransferase	70.20	30.07	1.23	3.59E−06
29014	Fructose-bisphosphate aldolase	829.19	364.65	1.19	3.55E−59
42447	Fructose-bisphosphate aldolase	1243.81	103.81	3.59	0.00E+00
41298	Glucose-6-phosphate isomerase	113.43	55.41	1.04	1.91E−04
29260	Transketolase	108.41	28.26	1.95	3.94E−27
32326	Ribose-phosphate pyrophosphokinase	50.95	21.01	1.29	5.52E−04
1769	Guanine deaminase	112.97	44.98	1.34	2.16E−09
15968	Xanthine dehydrogenase/oxidase	27.21	6.83	2.00	1.84E−13

Fold changes of transcripts are listed with a *P*-value <0.05. The values are the $\log_2$ expression ratio (PtAt/PtCtr).

this protein family requires further investigation for better understanding of the algal interaction.

3.5. Energy supply

3.5.1. Photosynthesis

Consistent with the TEM results for *P. tricornutum* (Fig. 2D and F), RNA-seq analysis showed that the genes associated with photosynthesis were downregulated, which affected the function of photosystem II (PSII) and photosynthetic electron transport. Similar results were also reported in *Chaetoceros neogracile* cells exposed to *A. minutum*, in which the red chlorophyll fluorescence, the activity of the photosynthetic reaction centers and the photosynthetic efficiency sharply decreased. The authors concluded that the primary putative allelopathic compounds were presented in the *A. minutum* supernatant [39].

Our results showed that the genes for PsbO (Phatr2_20331), PsbU (Phatr2_26293) and PsbM (Phatr2_55057), the subunits of PSII complex were downregulated in mixed cultures. This indicated that PSII activities were highly inhibited with respect to the oxygen evolving complex (OEC) and electron transport.

The ferredoxin-NADP+ reductase (Phatr2_42018; Phatr2_23717) and F-type ATPase gamma subunit (Phatr2_20657) were downregulated. Considering the rotation of gamma subunit found to induce cyclical conformational changes in the three alternating cooperative beta subunits and drive the ATP synthesis [40], the reduced expression of the ATPase gamma subunit might result in the suppression of ATP synthesis in co-cultivated *P. tricornutum*.

Furthermore, genes associated with chlorophyll and porphyrin metabolism were dramatically downregulated. Thirteen genes that were involved in the synthesis of chlorophyll a were downregulated by >4-fold, including chlorophyll synthase (Phatr2_12807, 18.81-fold). Genes encoding the enzymes associated with the xanthophyll cycle such as zeaxanthin epoxidase (Phatr2_45845), violaxanthin de-epoxidase (Phatr2_36048), eaxanthin epoxidase (Phatr2_45845) and violaxanthin de-epoxidase (Phatr2_36048), were also downregulated. The xanthophyll cycle was reported to play an essential role in photoprotection by synthesizing pigments such as diadinoxanthin and diatoxanthin [41], and also associated with the biosynthesis of fucoxanthin involved in the molecular acclimation to high light [42].

3.5.2. Oxidative phosphorylation

We identified that 10 genes were downregulated which were significantly associated with mitochondrial electron transport chain (from the mixed culture) including cytochrome bc_1 complex and ATP synthesis in *P. tricornutum*. In the cytochrome bc_1 complex, the ubiquinol-cytochrome *c* reductase iron-sulfur subunit (ISP, Phatr2_12233), ubiquinol-cytochrome *c* reductase cytochrome c_1 subunit (Cyt 1, Phatr2_26515) and ubiquinol-cytochrome *c* reductase subunit 7 (QCR7, Phatr2_46133) were downregulated. These three subunits catalyze the transfer of electrons coupled with protons across the membrane [43]. Furthermore, in the F-type ATPase, subunits such as alpha (Phatr2_14618), beta (Phatr2_54086), gamma (Phatr2_20657) and oligomycin sensitivity conferral protein (OSCP, Phatr2_44603) were also found to be downregulated along with inorganic pyrophosphatase (Phatr2_21183; Phatr2_32923; Phatr2_14529). Consequently, the suppression of the bc_1 complex and ATP synthesis in *P. tricornutum* mitochondria severely reduced the efficiency of electron transport chain and oxidative phosphorylation.

3.5.3. Carbohydrate metabolism

Mixed culture with *A. tamarense* induced the downregulation of the NAD-ME type in the C4 pathway due to the downregulation of mitochondrial enzymes such as aspartate aminotransferase (AAT_1, Phatr2_23059), NAD-dependent malic enzyme (NAD-ME, Phatr2_54082) and alanine aminotransferase (Phatr2_34010). Downregulation of these enzymes had a negative effect on efficiency of CO_2 delivery to Rubisco. However, a phosphoenolpyruvate carboxykinase (PEPCK1, Phatr2_55018) involved in the PEPCK type of the C4 pathway, was upregulated (2.24-fold) that could be a remedial action to balance the CO_2 fixation.

Moreover, genes involved in the carbon fixation of the Calvin cycle were also downregulated. The ribulose-phosphate 3-epimerase (PRE,

Phatr2_53935) and phosphoribulokinase (PRK, Phatr2_10208) located in the chloroplast stroma were both downregulated, thereby resulting in a decrease in ribulose-5-phosphate (RuP) and ribulose-1.5-bisphosphate (RuBP) expression. Furthermore, chloroplast stroma proteins, including fructose-1.6-bisphosphate aldolase (FbaC2, Phatr2_22993; FbaC1, Phatr2_825), fructose-1.6-bisphosphatase (FBPC3, Phatr2_9359) and phosphoglucomutase (PGM_1, Phatr2_32708), were also downregulated. Fructose-1.6-bisphosphatase could catalyze an irreversible breakdown of fructose-1.6-bisphosphate into fructose-6-phosphate and play a regulatory role in the pathways in which it is involved [44], we propose that CO_2 assimilation was reduced in *P. tricornutum*.

The results showed that genes associated with conversion of triose phosphates to pyruvate were downregulated. Of the three glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes identified in *P. tricornutum*, one was located in the cytosol (GAPDH_3, Phatr2_23598) and two were located in the mitochondria (TPI/GapC3, Phatr2_25308; GapC4, Phatr2_32747), and all these three genes were downregulated during mixed culture with *A. tamarense*, by 4.66-fold, 31.06-fold and 133.57-fold, respectively. Similarly, the transcription abundance of phosphoglycerate kinases (PGK) (Phatr2_29157, 16.77-fold; Phatr2_48983, 51.26-fold), and cytosolic pyruvate kinase (PK3, Phatr2_54998, 3.21-fold) were downregulated. Together, the significantly decreased transcript abundance of the genes involved in pyruvate metabolism in *P. tricornutum* could substantially decrease the production of pyruvate and carbon flux, thereby redirecting the carbon provision.

The components of the pyruvate dehydrogenase complex, linking glycolysis to the citric acid (TCA) cycle or other metabolic pathways, is thought to be a key regulator in the carbon flux of the TCA cycle [45]. Transcripts of this enzyme complex exhibited overall downregulation, suggesting the reduction of acetyl-CoA and suppression of the TCA cycle. Two pyruvate dehydrogenase E2 components (DHLTA_3, Phatr2_23850; Phatr2_1828) were dramatically downregulated by 33.79- and 6.32-fold, respectively. These results were similar to that of the pyruvate dehydrogenase E3 component, dihydrolipoamide dehydrogenase (pDLDH1, Phatr2_30113), with a 17.15-fold reduction. Dihydrolipoamide dehydrogenase is also a component of the 2-oxoglutarate dehydrogenase complex and play as a key control point of the TCA cycle [46]; thus, its downregulation resulted in the repression of carbon oxidation metabolism. Moreover, ATP-citrate synthase (ACL, Phatr2_54477), a rate-limiting enzyme catalyzing the first committed step in the TCA cycle, was also downregulated. However, two enzymes including aconitate hydratase 2 (Phatr2_26290, 19.34-fold) and isocitrate dehydrogenase (Phatr2_45017, 20.55-fold) involved in the TCA cycle were upregulated. The upregulation of several proteins associated with the TCA cycle has been observed in *A. thaliana* as a defense response to *Pseudomonas syringae* harpin [47], implying a requirement for increased energy metabolism during the defense response. Hence, the upregulation of these two enzymes in *P. tricornutum* might be a remedial action to regulate and balance the energy production in response to mixed culture with *A. tamarense*. Conversely, butyryl-CoA dehydrogenase (ACD1, Phatr2_25932) and acetyl-CoA C-acetyltransferase (Phatr2_23913) were upregulated by 10.61- and 2.35-fold, respectively. The upregulation of ACD1 and acetyl-CoA C-acetyltransferase in *P. tricornutum* implied that these enzymes played an anaplerotic role during acetyl-CoA deficiency through oxidation of short chain fatty acids, especially butanoyl-CoA, thus it regulated the TCA cycle regulating the provision of energy molecules.

Interestingly, two cytosolic fructose-bisphosphate aldolases (Fba3, Phatr2_29014; Fba4, Phatr2_42447) were upregulated by 2.29-fold and 12.05-fold, respectively, as well as the glucose-6-phosphate isomerase (GPI_3, Phatr2_41298). Therefore, production of cytosolic triose phosphates would be increased through glycolysis instead of further pyruvate formation. As described above, the genes involved in the conversion of triose phosphate in glycolysis and the Calvin cycle were both downregulated. However, given that glyceraldehyde-3-phosphate and fructose-1.6-bisphosphate were linked to the pentose phosphate pathway and this pathway was also thought to be upregulated on the basis of cytosolic transketolase (Phatr2_29260) and transaldolase (Phatr2_28222) upregulation, thus resulted in increased production of ribose-5-phosphate. Moreover, ribose-phosphate pyrophosphokinase (Phatr2_32326), which converts ribose-5-phosphate to phosphoribosyl pyrophosphate (PRPP), has been found to be upregulated, suggesting the accumulation of PRPP. However, a series of enzymes involved in the biosynthesis of DNA and/or mRNA, such as DNA polymerase delta subunit 2 (Phatr2_37723), DNA polymerase epsilon subunit 1 (Phatr2_52678) and DNA-directed RNA polymerase II subunit RPB7 (RNAP-II_3, Phatr2_35680), were downregulated, indicating that nucleic acids did not flow towards gene replication and transcription in *P. tricornutum* from the mixed culture with *A. tamarense*. Additionally, the transcript level of guanine deaminase (Phatr2_1769) and xanthine dehydrogenase/oxidase (Phatr2_15968) increased 2.5- and 4.0-fold, respectively, indicating that nucleic acid metabolism was susceptible to degradation and formation of urate. In *A. thaliana*, urate strongly reduced the cell death induced by peroxynitrite during the hypersensitive response against virulent *Pseudomonas syringae* [48]. The upregulation of urate synthetic pathway in *P. tricornutum* under mixed culture with *A. tamarense* therefore could contribute to cell death prevention and the stabilization of the cell.

3.6. Cell cycle

Some allelochemicals have been shown to inhibit mitosis and cell cycle [49,50]. In our data, the transcription levels of several proteins with key functions in the cell cycle of *P. tricornutum* were suppressed by mixed culture with *A. tamarense*. Phatr2_47264 (2.11-fold) was downregulated, which was annotated as transcription factor E2F2 and could play a role in activating the transition of G1/S phase [51]. Moreover, transcript level of some key genes encoding proteins in DNA biosynthesis such as minichromosome maintenance (MCM) complex and proliferating cell nuclear antigen (PCNA) were found to be downregulated. Three subunits, MCM2 (Phatr2_18622, 2.04-fold), MCM3 (Phatr2_51597, 2.53-fold) and MCM4 (Phatr2_51412, 2.33-fold) participated in the MCM complex were downregulated, indicating the suppression of both initiation and elongation of DNA replication forks [52]. Similarly, the downregulation of PCNA (Phatr2_29196, 2.56-fold) might inhibit the leading strand synthesis and affect DNA damage and repair responses [53]. Furthermore, the transcription level of Phatr2_9475 (2.17-fold), which was annotated as Cdc27, a subunit of anaphase-promoting complex (APC/C), also decreased; this implied the suppression of transition from metaphase to anaphase [54]. Together, the proteins associated with check point activation of cell division were downregulated, thereby suppressing mitosis and cell division in *P. tricornutum* during mixed culture with *A. tamarense*.

Our results do not concur with mitochondria-mediated PCD (programmed cell death) processes, since no characteristics of PCD such as the formation of apoptotic body were found under EM (Fig. 2), although it has been reported that mitochondria are sensitive to alleochemicals [55]. Vardi [56] reported that nitric-oxide (NO) could play an essential role in signaling PCD in *P. tricornutum*, however, in our study, there was so significant changes in the transcript level of genes associated with NO formation and urea cycle, such as arginine-dependent nitric-oxide synthase. Thus, we propose that NO-related pathways might not play a significant role in *P. tricornutum* during mixed culture. In *Trichodesmium* spp. under stress, some caspase-like proteins were found to be activated and mediated PCD of algal cells [57], while in our study genes encoding caspases did not show significantly different expression ($|\log_2$Fold-change$| < 1$).

4. Conclusions

The present study showed that the co-incubation of *A. tamarense* and *P. tricornutum* induced a negative effect on the growth of diatom cells,

with a significant reduction ($P < 0.05$) in cell density within 24 h. The transcription levels of genes involved in a range of metabolic pathways in *P. tricornutum* were affected (Fig. 6). Genes associated with photosynthesis, oxidation phosphorylation, glycolysis, the TCA cycle, carbon fixation and the cell cycle were downregulated, and several genes involved in the defense response such as ABC transporters and the predicted FLS2 protein family were upregulated. We speculated that there might be another cell-cell interaction pathway present between the diatom and the dinoflagellate, i.e., flagellin and the FLS2 receptor, which was distinct from the well-known allelopathy effect. Genome-scale transcriptome analysis conducted during the co-incubation of *P. tricornutum* and *A. tamarense* helped to unravel the responses of the diatom to the toxic dinoflagellate at the molecular level.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.04.019.

Author contributions

J.W.Z., D.W.L., Y.L., and J.C. performed the experimental analysis and contributed to the writing of the paper. J.W.Z., J.J.L., and L.Z. annotated the data. W.D.Y. provided technical expertise. J.S.L. and S.H.L. contributed to the text of the paper. H.Y.L. designed the study, coordinated and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Acknowledgments

This work was supported by the National Science Foundation of China (41230961, 41576132), the Guangdong Natural Science Foundation (2014A030308010) and the Department of Education of Guangdong (2014KTSCX022).

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